(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Veröffentlichungsnummer:
(11) Publication number:
(11) Numéro de publication:

0 625 190

Internationale Anmeldung veræffentlicht durch die
Weltorganisation fßr geistiges Eigentum unter der Nummer:

**WO 94/09114** (art.158 des EP*f*).

International application published by the World
Intellectual Property Organisation under number:

**WO 94/09114** (art.158 of the EPC).

Demande internationale publieà par l'Organisation
Mondiale de la Propriàtà sous le numàro:

**WO 94/09114** (art.158 de la CBE).

Express mail: EV 829030955 US
Entry into National Stage of PCT/EP2005/050577
Attorney Docket: I-2004.011 US

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 1/20, A61K 39/02, 39/295 A61K 39/116 // (C12N 1/20 C12R 1:01)

A1

(11) International Publication Number: WO 94/09114

(43) International Publication Date: 28 April 1994 (28.04.94)

(21) International Application Number: PCT/EP93/02873

(22) International Filing Date: 14 October 1993 (14.10.93)

(30) Priority data:
92203154.7 14 October 1992 (14.10.92) EP
*(34) Countries for which the regional or international application was filed:* NL et al.

(71) Applicant *(for all designated States except US):* AKZO NOBEL N.V. [NL/NL]; Velperweg 76, NL-6824 BM Arnhem (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* STORM, Paul, Karel [NL/NL]; De Raetsingel 14, NL-5831 KC Boxmeer (NL). VAN EMPEL, Paul, Cornelius, Maria [NL/NL]; Bakelgeertstraat 66a, NL-5831 CV Boxmeer (NL).

(74) Agent: HERMANS, F., G., M.; Postbus 20, NL-5340 BH Oss (NL).

(81) Designated States: HU, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**
*With international search report.*

(54) Title: NEW BACTERIUM CAUSING POULTRY DISEASE AND VACCINE DERIVED THEREOF

(57) Abstract

The present invention relates to a novel bacterial respiratory poultry disease and the identification of the causative agent. A vaccine derived from this agent was effective in preventing the disease in chickens challenged with the virulent field strains.

New bacterium causing poultry disease and vaccine derived thereof.

The present invention is concerned with a novel type of gram-negative aerobic rod-shaped bacterium, a vaccine derived thereof and with the use of a novel type of Gram-negative aerobic rod-like bacterium.

In the last decades, in many countries a strong raise in both the number of chicken and poultry farms, and in addition, an increasing number of animals per farm has been seen. This situation has a serious consequence: it has caused an increasing need for new and better vaccines and vaccination programmes in these countries. Nowadays, most animals are immunized against a number of diseases of viral, bacterial and parasitic origin. Examples of viral diseases in poultry are Newcastle Disease, Infectious Bronchitis, Turkey Rhinotracheitis, Herpesvirus of Turkeys, Fowlpox, Infectious Bursal Disease, etc. Examples of bacterial diseases are Coryza, Salmonella infections, Pasteurella multocida infections and E. coli infections.

A new bacterial respiratory disease has surprisingly been observed in chickens and turkeys.
The disease was seen in chickens that had been vaccinated against the bacterium Haemophilus paragallinarum; the causative agent of a disease called Coryza. Coryza is, as far as known, the only respiratory disease in chicken, caused by bacteria belonging to the families of Pasteurellaceae and Neisseriaceae.
The symptoms of this new disease differ from the specific symptoms of Coryza. Coryza is mainly an infection of the upper respiratory tract. Infected animals show a serous to mucoid nasal discharge, facial edema and conjunctivitis. They do however not show the clinical signs belonging to diseases of the lower

well. Accumulation of plasma cells and heterophils are noticeable with some multi-nuclear giant cells and granulomatous infiltrations. No specific micro-organisms are visible in sections with Ziehl-Nielsen and PAS staining. In live birds no pericarditis, perihepatitis or splenitis is usually seen.
From affected airsacs Pasteurella/Neisseria-like organisms were isolated.
These isolates did not seem to be classic species in the sense that they do in spite of their relatedness to Pasteurella and Neisseria, not belong to these species and some variation in their biochemical abilities has been noticed.

In turkey flocks in several parts of the world, a comparable infection of the upper respiratory tract was found. At first appearance, a low mortality was found, although at this moment mortality in flocks suffering from the disease can be as high as 5%.
The first clinical signs are comparable to infection in chicken: sneezing and nasal discharge. In some animals clinical signs of acute infection were seen. Examination of sacrificed animals showed edema of the lungs, fibrinopurulent pneumonia and often serofibrinous pericarditis and serofibrinous infection of the airsacs.

Bacteria were isolated from infected airsacs and purified.
After purification, isolates were grown on rich agar dishes in order to obtain large quantities of pure pathogen.
In order to check for the validity of the Koch postulates, a group of S(pecific) P(athogen) F(ree) animals was infected with a mixture of isolates. After infection, they all showed clinical signs that were indistinguishable from those, seen in field infections.

More in particular, the present invention is directed to gram-negative aerobic rod-shaped bacteria that positively react in an Agar Gel Precipitation test with antiserum derived against the deposited strain.

The deposited bacterium was typed according to standard determination methods, using Bergey's Manual of Systematic Bacteriology Volume 1 (1984, Williams and Wilkins, 428 East Preston Street, Baltimore U.S.A., 1984) and A.P.I SYSTEM, La Balme-les-Grottes 38390 Montalieu-Vercie, France, system numbers API 20E, API 20NE, API 50CHE, API ZYM, API OF.
Results are shown in table 1.

Table 1, differentiation tests.

| | |
|---|---|
| nitrate reduction | - |
| V-factor requirement | - |
| catalase | - |
| cytochrome-oxidase | + |
| growth on McConkey-agar | - |
| Voges Proskauertest (37°C) | + (weakly) |
| Urease | + |
| lysine decarboxylase | - |
| ornithine decarboxylase | - |
| O.N.P.G. or P.N.P.G. (ß-gal) | + |
| strictly aerobic | - |
| arginine dehydrolase | + (temp.-dependent) |
| indole | - |
| fermentation of: | |
| fructose | + |
| lactose | + |
| galactose | + |

The combination of characteristic properties as given in table 1 makes the novel type of bacteria unique compared to other known bacterial poultry pathogens. (Diseases of Poultry 8, Iowa State University Press 1984).
Incidentally, a strain according to the invention may react negatively in a test of table 1, where the deposited strain reacts positively, or vice versa. This

repeated vaccination in the presence of adjuvant. Strain GGD 1261 is a strain, recently isolated from turkeys by Dr H.M. Hafez, State Veterinary Laboratory of Stuttgart Germany). As is clearly shown in table 3, all strains are (cross-)reactive, although strains originating from chickens react better with antisera against chicken strains, and the turkey-strain react better with antisera against the turkey strain.

It is obvious, that any strain isolatable from airsacs of animals suffering from the described illness and serologically related to the deposit strain also falls within the scope of the present invention.
Thus, the novel type of bacterium comprises bacteria which are cross-reactive with the deposited bacterial strain, i.e. serum raised against a novel type bacterium binds to the deposited bacterium and vice versa.
In order to discriminate between the novel type of bacterium of the present invention and other gram-negative aerobic rod-shaped bacteria, two serological tests were done:
a) the strain of the present invention was tested in an Agar Gel Precipitation test according to Heddlestone (Heddleston, K.L. et al. (Avian Diseases 16: 925 (1972)) against strain 3037/91, strain 3290/91(A), strain 3290/91(K), all isolated from chickens, and strain GGD-1261, isolated from turkey. In all cases, crossreaction was found.
The strain of the present invention was also tested with Haemophilus paragallinarum strains H18, Spross, 0083, against Kingella kingae, and Kingella denitrificans, against Suttonella indologenes, against Pasteurella gallinarum, against the known 16 serotypes of Pasteurella multocida and against 10 serotypes of Pasteurella anatipestifer. No cross-reactivity was found.
b) The strains mentioned in Table 3 were tested in an ELISA assay against three different serotypes of Haemophilus paragallinarum, against two Kingella strains, against Suttonella indologenes and against Pasteurella gallinarum. The results, given in table 4 show that, although the cross-reactivity between related strains is (very) high, there is no cross-reactivity between any of the strains from table 3 and the known strains listed in table 4.

Table 4b: SEROLOGICAL RESPONSES OF THE COMBINED SERA OF THE GROUPS AGAINST BOILED CAPSULAR EXTRACTS SERUM TAKEN 3 WEEKS AFTER 2nd VACCINATION.
Sera with a titer of 10 or >10 are considered to belong to to the same serotype.

| SERUM NR | VACC. WITH | TITRE (IN 2 LOG) AGAINST B.C.A. OF STRAIN; | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Hpg-H18 | Hpg-SPROSS | Hpg-0083 | K. kingae | K. denitr. | S. indolog. | P gallin. |
| 612 | control | <6 | <6 | <8 | <6 | 8 | 6 | <6 |
| 613 | 3037/91 | 7 | 6 | 6 | <9 | <9 | <9 | 6 |
| 614 | 3263/91 | <6 | 6 | <6 | <9 | <9 | <9 | 6 |
| 615 | 3290/91(A) | 7 | 8 | 7 | <9 | <9 | <9 | 7 |
| 618 | 3290/91(K) | 6 | 8 | 6 | <9 | <9 | <9 | 7 |
| 619 | GGD-1261 | 6 | 7 | 6 | <6 | 7 | 7 | 7 |
| 620 | Hpg-H18 | >13 | 13 | 10 | 8 | 12 | 11 | 9 |
| 621 | Hpg-Spross | 11 | >13 | >13 | 8 | 12 | 11 | 9 |
| 622 | Hpg-0083 | 11 | 13 | >13 | 9 | 11 | 11 | 8 |
| 628 | K. kingae | 8 | 9 | 7 | >15 | 15 | 7 | 7 |
| 629 | K. denitr. | 9 | 9 | 7 | 12 | >15 | 7 | 7 |
| 630 | S. indolog. | 6 | 6 | <6 | 6 | 6 | >15 | 6 |
| 631 | P. gallin. | 8 | 9 | 8 | 8 | 8 | 8 | >13 |

as the relevant immunogen in the vaccine according to the invention.

In a preferred embodiment, said vaccine comprises inactivated bacteria.
Various physical and chemical methods of inactivation are known in the art. Examples of physical inactivation are UV-radiation, X-ray radiation, gamma-radiation and heating. Examples of inactivating chemicals are ß-propiolactone, glutaraldehyde, ethyleneimine and formaldehyde.

Preferably the strain is inactivated with formaldehyde. It is obvious that other ways of inactivating the bacteria are also embodied in the present invention.

The vaccine according to the invention in a preferred presentation also comprises an adjuvant. Adjuvants in general comprise substances that boost the immune response of the injected animal. A number of different adjuvants are known in the art. Examples of adjuvants are Freunds Complete and Incomplete adjuvant, vitamin E, non-ionic block polymers, muramyldipeptides, Quill A, mineral oil, vegetable oil, and Carbopol (a homopolymer). In addition, the vaccine may comprise one or more suitable emulsifiers, e.g. Span or Tween.

In a preferred embodiment, the bacterin comprises a water-in-oil emulsion adjuvant.
It goes without saying, that other ways of adjuvating the bacteria are also embodied in the present invention.

The vaccine in the present invention contains at least one antigen of a bacterium of the novel type characterised by the bacterium deposited under CBS 400.92. This includes whole cells, bacterial extracts,

3290/91(K) were grown on sheepblood-agar for 48 hours at $37^{o}$ C. with the use of a Gas-pac system in order to obtain a 5-10% $CO_2$ environment. Cells were washed off and a C(olony) F(orming) U(nits) count was performed. Cells were killed by adding formaldehyde to a final concentration of 0.185%. After a sterility-check, cells were diluted to $5*10^8$ C.F.U./cell-type in 1 ml of the final vaccine.

The vaccine was prepared by mixing the four strains and oil-adjuvant (a water-in-oil emulsion on the basis of a mineral oil with a ratio of 55% oil / 45% water) to a final concentration of $5*10^8$ cells/strain/ml.

Vaccination was done in broilers at ten days of age and was performed by injection of 0.5 ml of the vaccine subcutaneously halfway the neck.

## EXAMPLE II

### Preparation of challenge strains and challenge of vaccinated and control groups.

Preparation 1): bacterial strains 3037/91, 3263/91, 3290/91(A) and 3290/91(K) were grown in Brain Heart Infusion broth, for 20 hrs at $37^{o}$ C. For challenge, preparations were made that contain the following number of cells in the final challenge-volume:

$3.4*10^8$ c.f.u. of strain 3037/91
$2.2*10^8$ c.f.u. of strain 3263/91
$3.4*10^8$ c.f.u. of strain 3290/91(A)
$7.0*10^7$ c.f.u. of strain 3290/91(K)

virulence of pathogenic strains, and also for the efficacy of vaccination.
The strain was grown on egg-yolk as described under EXAMPLE II: preparation of challenge strains. Challenge material was brought directly into the airsacs, in a concentration of $1.2 * 10^8$ C.F.U. per animal.

Table 5: Comparison of growth-development in chickens infected with live strain 3262/91 and control group.

| Challenge | strain 3263/91 | control group |
|---|---|---|
| Average weight day 0 | 1100 (± 98) | 1143 (± 110) |
| Average weight day 8 | 1179 (± 132) | 1478 (± 92) |
| Average weight day 14 | 1684 (± 162) | 1935 (± 91) |
| Average weight diff. day 0-8 | 93 (± 114)[a] | 314 (± 64) |
| Average weight diff. day 0-14 | 600 (± 165)[b] | 796 (± 74) |

a= significantly different from the control group, $p<0.005$
b= significantly different from the control group, $p<0.05$

B) Virulence of strain 3263/91 and GGD 1261 in turkeys.
The table 6 given below shows the virulence of strain 3263/91 deposited under CBS 400.92 and the turkey strain GGD 1261 in turkeys, determined by growth-retardation, when they are used as live challenge-strains. The strains were grown on egg-yolk as described under EXAMPLE II: preparation of challenge strains. Challenge material was brought directly into the airsacs, in a concentration of $5 * 10^8$ C.F.U. per animal at an age of 32 days. Eleven days after the infection, the turkeys were sacrificed.

D) Vaccination-challenge experiments in relation with daily weigth-gain.

In table 7, the average daily weight gain of chickens over a period of 34 days is given. It is easily seen from this table on the basis of differences in daily weight gain, that turkey strain GGD 1261 is pathogenic for chickens. Most important however is the notice, that vaccination with the deposited strain 3263/91 gives protection against GGD-1261 challenge.

Table 7: vaccination challenge experiments in chickens with vaccines based on strain 3263/91 and GGD 1261

| GROUP | AVG | STD | n |
|---|---|---|---|
| 1) control | 60 | 14 | 10 |
| 2) chall GGD-1261 | 42 | 9 | 10 |
| 3) vacc. GGD-1261 and hom. chall. | 61 | 24 | 10 |
| 4) vacc 3263/91 + chall GGD-1261 | 60 | 12 | 10 |

| GROUPS | P= |
|---|---|
| Group 1 vs Group 2 | <0.005 |
| Group 1 vs Group 3 | >0.05 |
| Group 2 vs Group 3 | <0.025 |
| Group 2 vs Group 4 | <0.005 |

AVG=average, STD=standard deviation, n= number of animals.

CLAIMS

1) Gram-negative aerobic rod-shaped bacterium of a novel type, characterised by a bacterium deposited at the Centraalbureau voor Schimmelcultures under deposit number 400.92.

2) Bacterium of claim 1, characterised in that the bacterium is of the strain deposited at the Centraalbureau voor Schimmelcultures under deposit number 400.92.

3) Microbiological culture comprising a bacterium, characterised in that the culture comprises a bacterium according to claim 1 or 2.

4) Vaccine effective against respiratory diseases in poultry, characterised in that it is derived from bacteria according to claim 1 or 2.

5) Vaccine according to claim 4, characterised in that the bacteria are inactivated.

6) Vaccine according to claim 4 or 5, characterised in that the vaccine also comprises an adjuvant.

7) Vaccine according to claim 4-6, characterised in that it further comprises at least one other antigen from a virus or micro-organism pathogenic to poultry.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Intervet International B.V.
P.O.Box 31
5830 AA BOXMEER
The Netherlands

*name and address of depositor*

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

**I. IDENTIFICATION OF THE MICROORGANISM**

| Identification reference given by the DEPOSITOR: | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: |
|---|---|
| 3263/91 | CBS 400.92 |

**II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

The microorganism identified under I above was accompanied by:

[X] a scientific description

[X] a proposed taxonomic designation

*(mark with a cross where applicable)*

**III. RECEIPT AND ACCEPTANCE**

This International Depositary accepts the microorganism identified under I above, which was received by it on Tuesday, 8 September 1992 *(date of the original deposit)*[1]

**IV. RECEIPT OF REQUEST FOR CONVERSION**

The microorganism identified under I above was received by this International Depositary Authority on not applicable *(date of the original deposit)* and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on not applicable *(date of receipt of request for conversion)*

**V. INTERNATIONAL DEPOSITARY AUTHORITY**

Name: Centraalbureau voor Schimmelcultures

Address: Oosterstraat 1
P.O. Box 273
3740 AG BAARN
The Netherlands

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

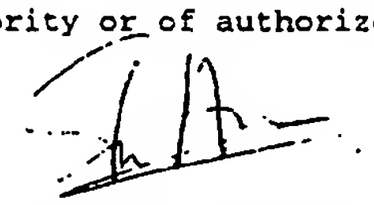
drs F.M. van Asma

Date: Monday, 12 October 1992

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page) CBS/9107

| IV. CONDITIONS UNDER WHICH THE VIABILITY HAS BEEN PERFORMED [4] |
|---|
| |

| V. INTERNATIONAL DEPOSITARY AUTHORITY | |
|---|---|
| Name: Centraalbureau voor Schimmelcultures<br><br>Address: Oosterstraat 1<br>P.O. Box 273<br>3740 AG BAARN<br>The Netherlands | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):<br>  <br>drs F.M. van Asma<br><br>Date: Monday, 12 October 1992 |

[4] Fill in if the information has been requested and if the results of the test were negative.

Form BP/9 (second and last page)

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir tional Application No
PCT/EP 93/02873

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US-A-3534136 | 13-10-70 | NONE | |
| US-A-3876763 | 08-04-75 | BE-A- 777498 | 17-04-72 |
| | | DE-A,B,C 2165401 | 13-07-72 |
| | | FR-A,B 2120078 | 11-08-72 |
| | | GB-A- 1324618 | 25-07-73 |
| | | GB-A- 1324619 | 25-07-73 |
| | | NL-A- 7117873 | 03-07-72 |
| | | SE-B- 398514 | 27-12-77 |